A Pleckstrin Homology Domain Specific for Phosphatidylinositol 4,5-Bisphosphate (PtdIns-4,5-P$_2$) and Fused to Green Fluorescent Protein Identifies Plasma Membrane PtdIns-4,5-P$_2$ as Being Important in Exocytosis*

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Kinetically distinct steps can be distinguished in the secretory response from neuroendocrine cells with slow ATP-dependent priming steps preceding the triggering of exocytosis by Ca^{2+}. One of these priming steps involves the maintenance of phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-P$_2$) through lipid kinases and is responsible for at least 70% of the ATP-dependent secretion observed in digitonin-permeabilized chromaffin cells. PtdIns-4,5-P$_2$ is usually thought to reside on the plasma membrane. However, because phosphatidylinositol 4-kinase is an integral chromaffin granule membrane protein, PtdIns-4,5-P$_2$ important in exocytosis may reside on the chromaffin granule membrane. In the present study we have investigated the localization of PtdIns-4,5-P$_2$ that is involved in exocytosis by transiently expressing in chromaffin cells a pleckstrin homology (PH) domain that specifically binds PtdIns-4,5-P$_2$ and is fused to green fluorescent protein (GFP). The PH-GFP protein predominantly associated with the plasma membrane in chromaffin cells without any detectable association with chromaffin granules. Rhodamine-neomycin, which also binds to PtdIns-4,5-P$_2$, showed a similar subcellular localization. The transiently expressed PH-GFP inhibited exocytosis as measured by both biochemical and electrophysiological techniques. The results indicate that the inhibition was at a step after Ca^{2+} entry and suggest that plasma membrane PtdIns-4,5-P$_2$ is important for exocytosis. Expression of PH-GFP also reduced calcium currents, raising the possibility that PtdIns-4,5-P$_2$ in some manner alters calcium channel function in chromaffin cells.

The importance of inositol phospholipids, especially the polyphosphoinositides, in cell function was first recognized be-

cause of their involvement in cell signaling as substrates for phospholipase C with the resulting production of Ins(1,4,5)P$_3$ [1] and diacylglycerol (1–3). It was subsequently found that the PtdIns-4,5-P$_2$ could directly interact with and regulate the function of several cytoskeletal proteins (4–7), indicating the lipids are likely to be able to regulate other complex cellular functions. Evidence has since accumulated that the polyphosphoinositides interact with specific proteins in a variety of vesicular trafficking pathways including exocytosis and endocytosis and are necessary for the proper functioning of these pathway (for reviews, see Refs. 8–10).

The first evidence that polyphosphoinositides play an important role in vesicular trafficking reactions came from studies of regulated exocytosis in chromaffin cells (11). Studies in permeabilized chromaffin cells demonstrated that the secretory pathway could be separated into distinct kinetic steps with different biochemical characteristics (12–14). A slow ATP-dependent priming step preceded a rapid Ca^{2+}-dependent triggering step. Subsequent studies demonstrated that a major component of the ATP dependence of secretion reflected the maintenance of the polyphosphoinositides. The enzymatic removal of phosphatidylinositol in permeabilized cells resulted in the subsequent decline in PtdIns-4,5-P$_2$ and PtdIns-4-P and the specific inhibition of ATP-dependent secretion (11). Studies in PC12 cells strongly advanced the concept. Two cytosolic factors that were necessary for ATP-dependent priming of exocytosis were identified as a phosphatidylinositol transfer protein (15) and phosphatidylinositol 4-phosphate kinase (16). These studies directly implicated PtdIns-4,5-P$_2$. Polyphosphoinositides have also been implicated in synaptic vesicle exocytosis (17, 18), endocytic recycling of synaptic vesicle membrane (8), and endocytosis of G protein-coupled receptors (19). Polyphosphoinositides labeled in the 3-position of the inositol ring are necessary for vesicular trafficking between the Golgi and vacuole/lysosome in yeast and mammalian cells (10, 20).

PtdIns-4,5-P$_2$ is located on the plasma membrane (21–23). However, it has also long been known that PtdIns 4-kinase, one of the key enzymes in PtdIns-4,5-P$_2$ synthesis, is an integral

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[1] The abbreviations used are: Ins(1,4,5)P$_3$, inositol 1,4,5-trisphosphate; DMPP, dimethylphenylpiperazinium, a nicotinic agonist; GFP, green fluorescent protein; hGH, human growth hormone; KGEP, solution containing potassium glutamate, EGTA, and PIPES; PH, pleckstrin homology domain; PSS, physiological salt solution; PIPES, 1,4-piperazinediethanesulfonic acid; PtdIns, phosphatidylinositol.

membrane protein of the chromaffin granule membrane (24–27). PtdIns 4-kinase is also associated with synaptic vesicles (17) and mast cell granules (28). It is, therefore, possible that PtdIns-4,5-P$_2$ could be synthesized on the secretory granule membrane through the sequential action of granule PtdIns 4-kinase and PtdIns-4-P 5-kinase.

There are several chromaffin granule membrane proteins that have been implicated in exocytosis that specifically bind PtdIns-4,5-P$_2$: synaptotagmin (29), calcium-dependent activator protein for secretion (30), and Rabphilin3 (31). If the interaction of one or more of the proteins with PtdIns-4,5-P$_2$ is involved in secretion, there will be mechanistic consequences of the localization of PtdIns-4,5-P$_2$.

In the present study we have investigated the localization of PtdIns-4,5-P$_2$ that is involved in exocytosis by transiently expressing in chromaffin cells a pleckstrin homology (PH) domain that specifically binds PtdIns-4,5-P$_2$ and is fused to green fluorescent protein (GFP). PH domains are approximately 100-amino acid motifs with a common tertiary structure with little sequence homology and different binding activities (for reviews, see Refs. 32 and 33). The PH domain of phospholipase Cδ_1 is responsible for the binding of the enzyme to its substrate, PtdIns-4,5-P$_2$, on the plasma membrane (34). The isolated PH domain binds specifically and with high affinity to PtdIns-4,5-P$_2$ and to Ins(1,4,5)P$_3$ (35–37) because of strong interactions between PH domain residues and the 4-and 5-phosphates on the inositol ring (38). A protein ligand for PH δ_1 has not been identified. Recently, the phospholipase C δ_1 PH domain fused to GFP (PH-GFP) was used to visualize PtdIns-4,5-P$_2$ in cells (39, 40). The transiently expressed protein was predominantly localized to the plasma membrane in unstimulated NIH-3T3, COS-7, and adrenal glomerulosa cells, but translocated to the cytosol upon stimulation of phospholipase C. There was a strong correlation between the translocation and the loss of PtdIns-4,5-P$_2$ (39). We have transiently expressed the same fusion protein in bovine adrenal chromaffin cells. We demonstrate that it predominantly associates with the plasma membrane without any detectable association with chromaffin granules. Localization of PtdIns-4,5-P$_2$ was also investigated with rhodamine-labeled neomycin. Neomycin is an aminoglycoside antibiotic that strongly binds PtdIns-4–5-P$_2$ (41–43). Rhodamine-neomycin also labeled the plasma membrane. Transiently expressed PH-GFP specifically inhibited exocytosis in intact and permeabilized cells, indicating that plasma membrane PtdIns-4,5-P$_2$ is important for exocytosis.

MATERIALS AND METHODS

Chromaffin Cell Preparation, Transfection, and Secretion Experiments—Chromaffin cell preparation, transient transfection, and human growth hormone secretion experiments were performed as described previously (44, 45). Ca^{2+} phosphate precipitation was used for transfections according to Wilson *et al.* (46) in 12-well plates (22.6-mm well diameter) for secretion experiments. Human growth hormone secretion experiments were generally performed 5–6 days after transfection at 27 °C. Intact cell experiments were performed in a physiological salt solution (PSS) containing 145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl$_2$, 0.5 mM MgCl$_2$, 5.6 mM glucose, and 15 mM HEPES (pH 7.4). Secretion from permeabilized cells were performed in potassium glutamate solution (KGEP) containing 139 mM potassium glutamate, 20 mM PIPES (pH 6.6), 2 mM MgATP, 20 μM digitonin, and 5 mM EGTA with either no added Ca^{2+} or sufficient Ca^{2+} to yield 30 μM buffered Ca^{2+}. Methoxyverapamil (10 μM D-600) was included in the KGEP to inhibit Ca^{2+} influx through voltage-sensitive Ca^{2+} channels that could possibly stimulate secretion from poorly permeabilized cells. There were four wells or dishes/group. Human growth hormone (hGH) was measured with a high sensitivity chemiluminescence assay from Nichols Institute (San Juan Capistrano, CA). Endogenous catecholamine secretion was measured with a fluorescence assay (47). Since only approximately 2% of the cells are transfected, catecholamine secretion mainly reflects secretion from nontransfected cells and served as a control in the hGH

secretion experiments. Secretion was expressed as the percentage of the total cellular hGH (or catecholamine) that was released into the medium. There was usually 0.5–2.0 ng of hGH and 20–40 nmol of catecholamine/22.6-mm diameter well.

Plasmids—The plasmids encoding the PH domain of phospholipase Cδ_1 fused to GFP (PH-GFP) or the mutant PH(S34T, R40L)-GFP were constructed as described previously (39).

Confocal Microscopy and Immunocytochemistry—Chromaffin cells were plated on glass coverslips (Fisher, no. 1 thickness) fastened to the bottom of punched-out wells on 12-well culture dishes (well diameter, 22.6 mm). Coverslips were sequentially coated with poly-D-lysine and calf skin collagen to promote cell adhesion. Cells were visualized with a Bio-Rad MRC600 laser scanning confocal microscope with a 100× objective (numerical aperture, 1.4) with a pin-hole aperture setting of 6–11, depending upon the nature of the experiment. In experiments in which the cytosolic intensity of PH-GFP was measured, the aperture setting was generally 8–11, which reduced the confocality but permitted sampling of a sufficient depth in the cytosol to obtain significant intensities of the weak cytosolic fluorescence. Neutral density filters was used reduce light intensity to a level that was just sufficient to obtain satisfactory images. Average pixel intensities were obtained of cytosolic areas that did not include the nucleus and of outlined membrane segments using Adobe Photoshop 4.0. In a given cell, average pixel intensities of the same cytosolic and membrane segments were determined in a sequence of timed images.

Electrophysiological Recording of I_{Ca} and C_m—Patch electrodes were pulled from 1.5 mm (outer diameter) × 1.12 mm (inner diameter) borosilicate glass (Corning 7740) capillaries (A-M Systems, Carlsborg, WA), coated with Sylgard elastomer (Dow Corning, Midland, MI), and fire-polished to 3–5 μm. The extracellular recording solution consisted of (mM): TEACl, 135; CaCl$_2$, 10; MgCl$_2$, 1; glucose, 10; and HEPES, 10 (pH 7.3 at room temperature). Electrodes were filled with a solution consisting of (mM): CsOH, 120; CH$_4$O$_3$S, 120; CsCl, 20; MgCl$_2$, 1; Mg-ATP, 2; Li-GTP, 0.5; EGTA, 0.25; and HEPES, 20 (pH 7.3). Standard whole cell patch-clamp recordings of calcium current (I_{Ca}) and membrane capacitance (C_m) were made using a modified Axopatch 200A amplifier (Axon Instruments, Burlingame, CA) with an ITC-16 computer interface (Instrutech Corp., Great Neck, NY). Voltage protocols, data acquisition, and analyses were performed using Pulse Control software (Drs. Jack Herrington and Richard Bookman, University of Miami Medical School, Miami, FL) developed as an extension of the numerical/graphics program Igor (WaveMetrics, Lake Oswego, OR). C_m measurements were made using a phase-tracking algorithm. To elicit I_{Ca} and changes in C_m, 50-ms step depolarizations from a holding potential of −90 mV to a test potential of +20 mV were applied to cells voltage-clamped at −90 mV. The standard stimulus protocol consisted of a train of eight such depolarizations with an interpulse interval of 200 ms. All recordings were made at room temperature. Identification of transfected cells was accomplished by co-transfection with a plasmid encoding ANP-emeraldGFP (generously provided by Dr. Edwin Levitan). ANP-emeraldGFP is directed to the regulated exocytotic pathway and packaged into secretory granules.

Synthesis of Rhodamine-Neomycin—Neomycin trisulfate (110 mg, 0.121 mmol) and rhodamine B isothiocyanate (8.2 mg, 0.015 mmol) were dissolved in 2 ml of 1.0 M triethylammonium bicarbonate (pH 8.6) and 0.2 ml of N,N-dimethylformamide. The solution was stirred 72 h at 25 °C. The solution was concentrated *in vacuo*, the residue was dissolved in water (10 ml), and byproducts were removed with two 10-ml methylene chloride extractions. The aqueous solution was again concentrated *in vacuo*, and reversed-phase (C$_{18}$) high pressure liquid chromatography fractions containing purified rhodamine-neomycin were collected using an acetonitrile gradient in 0.06% trifluoroacetic acid, and were lyophilized. Electrospray-mass spectrometry revealed an M^{+2} peak for the tetraprotonated species: m/z 511.4 (calculated for M + 4H, 1023.3).

RESULTS

PH-GFP Specifically Labeled the Plasma Membrane and Not Chromaffin Granules—Transiently expressed PH-GFP specifically labeled the plasma membrane in chromaffin cells (Fig. 1A). Arg-40 in the PH domain is one of three critical basic residues that interacts with PtdIns-4–5-P$_2$ (38). A PH domain with Arg-40 mutated to leucine (PH(S34T,R40L) fused to GFP) did not label the plasma membrane (Fig. 1B). To determine the localization of chromaffin granules in PH-GFP-expressing cells, chromaffin cells were transiently co-transfected with

FIG. 1. **PH-GFP specifically localizes to plasma membrane.** Chromaffin cells were transfected with a plasmid encoding wild type PH-GFP or a mutant PH(S34T, R40L)-GFP. Four days later the cultures were examined by confocal microscopy. The calibration *bar* is 5 μm.
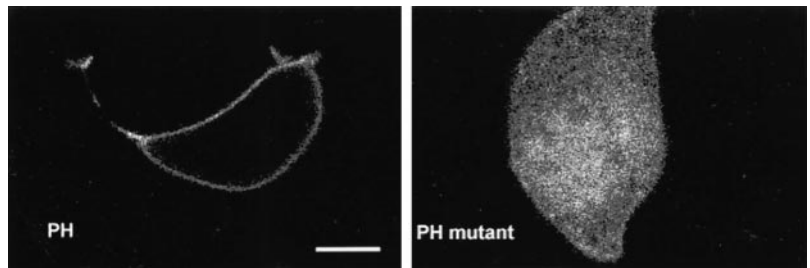
plasmids encoding PH-GFP and hGH. hGH is sorted to chromaffin granules (44). Immunocytochemistry was used to detect hGH and the intrinsic fluorescence of GFP to detect PH-GFP. Punctate hGH in chromaffin granules was distributed throughout the cell as is normally observed in cultured chromaffin cells (Fig. 2). PH-GFP did not co-localize with any of the granules and was again apparent on the plasma membrane. Some PH-GFP was also observed on the nuclear membrane.

Rhodamine-Neomycin Also Labels the Plasma Membrane— To further investigate the localization of PtdIns-4,5-P$_2$ in chromaffin cells, we examined the distribution of rhodamine-neomycin in chromaffin cells. Neomycin, which is an aminoglycoside antibiotic with a large positive charge ($\sim+4.5$), binds PtdIns-4,5-P$_2$ with high affinity (10^5 M^{-1}) (41–43). If PH-GFP labels the plasma membrane because of the presence of PtdIns-4,5-P$_2$, then rhodamine-neomycin should also label the plasma membrane. Rhodamine-neomycin may also reveal PtdIns-4–5-P$_2$ pools not accessible to the much larger PH-GFP. Because neomycin is not membrane-permeable, the experiments were performed in digitonin-permeabilized chromaffin cells. The *left side* of Fig. 3 shows bright field images, and the *right side* shows rhodamine fluorescence. When cells were permeabilized in the presence of ATP, the plasma membrane was highlighted (Fig. 3, *right side*). Other structures were also labeled including the nuclear membrane and nuclear structures. However, staining of multiple, small punctate structures in the cytoplasm that would reflect binding to chromaffin granules was not evident. ATP is required to maintain the levels of the polyphosphoinositides in chromaffin cells (11). The plasma membrane of cells permeabilized in the absence of ATP was not distinctly labeled by rhodamine-neomycin.

Stimulation of Chromaffin Cells Causes Shifts in the Distribution of PH-GFP—If the association of PH-GFP with the plasma membrane reflects binding of the protein to PtdIns-4,5-P$_2$, then increasing the turnover of PtdIns-4,5-P$_2$ by activation phospholipase C might directly or indirectly alter the distribution of PH-GFP. Angiotensin II, acting through a G protein-linked receptor, activates phospholipase C, hydrolysis of PtdIns-4,5-P$_2$, and production of Ins(1,4,5)P$_3$. The resulting submicromolar rise in cytosolic calcium is not large enough to stimulate significant secretion (2% or less of total cellular content of catecholamine is secreted; data not shown). The nicotinic agonist DMPP causes Ca^{2+} influx, an increase in cytosolic Ca^{2+} to micromolar or higher, and substantial secretion (15–30% of cellular catecholamine). The rise in cytosolic Ca^{2+} by DMPP also activates phospholipase C. Both effects of DMPP require calcium influx from the medium. Angiotensin II and DMPP each caused partial shifts of PH-GFP from the plasma membrane to the cytosol (Fig. 4). DMPP also caused changes in the peripheral pattern of PH-GFP with hot spots (*arrows*) that may reflect invaginations of the plasma membrane and/or regions of intense PtdIns-4,5-P$_2$ resynthesis. Angiotensin II caused severalfold increases in cytosolic PH-GFP and decreases in plasma membrane PH-GFP (Fig. 5, three cells). The responses were rapid and apparent by 10 s. Similar results were obtained with cells stimulated with DMPP in the presence
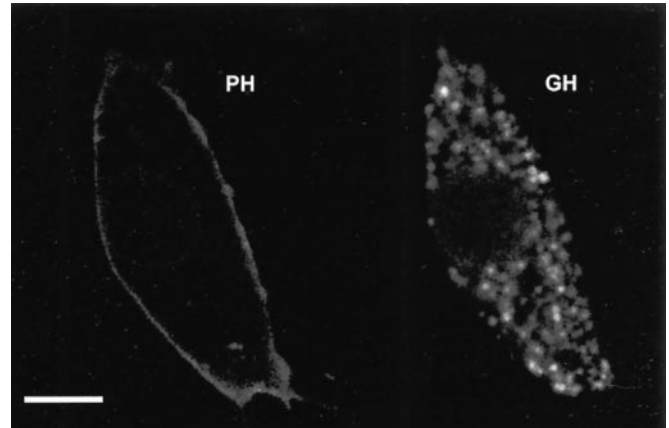

FIG. 2. **PH-GFP does not label secretory granules.** Chromaffin cells were co-transfected with plasmids encoding wild type PH-GFP and hGH. Five days later cells were fixed and permeabilized. Immuncytochemistry with anti-hGH identified secretory granules and fluorescence of GFP identified the PH domain. The calibration *bar* in the confocal image is 5 μm.

of Ca^{2+} (only the increase is cytosolic PH-GFP is shown since changes in the plasma membrane intensity could be confounded by membrane addition through exocytosis). The intensity of cytosolic PH-GFP increased 50–300% within 15 s. The DMPP-induced changes were not observed in calcium-free medium (Fig. 6, *lower panel*), indicating that Ca^{2+} influx is necessary for the DMPP-induced increases in PH-GFP.

To determine whether the changes in PH-GFP might reflect decreases in plasma membrane PtdIns-4,5-P$_2$, phosphoinositides in untransfected chromaffin cells were pre-labeled with ^{32}PO$_4$ and then stimulated (Table I). Angiotensin II caused only a small decrease (10–20%) in the polyphosphoinositides by 15 s. In another experiment there was no change (data not shown). After 2 min of stimulation (using a slightly different protocol than that used to stimulate at 15 s), there was no decrease caused by angiotensin II. DMPP did not decrease the polyphosphoinositides at either 15 s or 2 min. Instead, DMPP increased the level of PtdIns-4,5-P$_2$ by 30% and the level of PtdIns-4-P by 50% at 2 min. Ca^{2+}-dependent increases in the polyphosphoinositides caused by DMPP in intact cells and in permeabilized cells have been previously observed (48) and are probably caused, at least in part by a Ca^{2+}-dependent increase in lipid phosphorylation.

Because of the low transfection rates (2% or fewer of the cells), phospholipid metabolism could not be directly measured in transfected cells. Nevertheless, the experiments suggest that the partial shifts in localization of PH-GFP induced by angiotensin II and DMPP are unlikely to be caused by decreases in PtdIns-4,5-P$_2$. Because the PH domain has at least as high affinity for Ins(1,4,5)P$_3$ as for PtdIns-4,5-P$_2$ (35, 49, 50), it is possible that the shifts are caused by the production of Ins(1,4,5)P$_3$ and perhaps other inositol polyphosphates. To investigate the importance of activation of phospholipase C in shifts in localization of PH-GFP, cells expressing PH-GFP were

FIG. 3. **Rhodamine-neomycin labels the plasma membrane in digitonin-permeabilized chromaffin cells.** *A*, nontransfected chromaffin cells were permeabilized with Ca^{2+}-free KGEP containing 2 mM MgATP and 20 μM digitonin. After 4 min, the solution was replaced with Ca^{2+}-free KGEP containing 2 mM MgATP and 10 μM rhodamine-neomycin but without digitonin. The confocal image was taken 15 min later. *B*, nontransfected chromaffin cells were permeabilized with Ca^{2+}-free and ATP-free KGEP with 20 μM digitonin. After 6 min the solution was replaced with Ca^{2+}-free and ATP-free KGEP with rhodamine-neomycin without digitonin. The confocal image was taken 11.5 min later. The *left panels* are bright field, and the *right panels* are rhodamine-neomycin fluorescence. Note that rhodamine-neomycin labeled the cell periphery in the presence of ATP but not in its absence. The calibration *bar* is 5 μm.
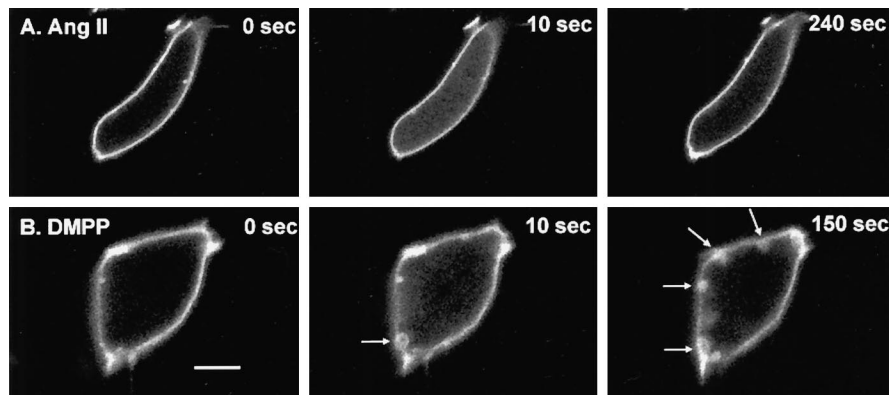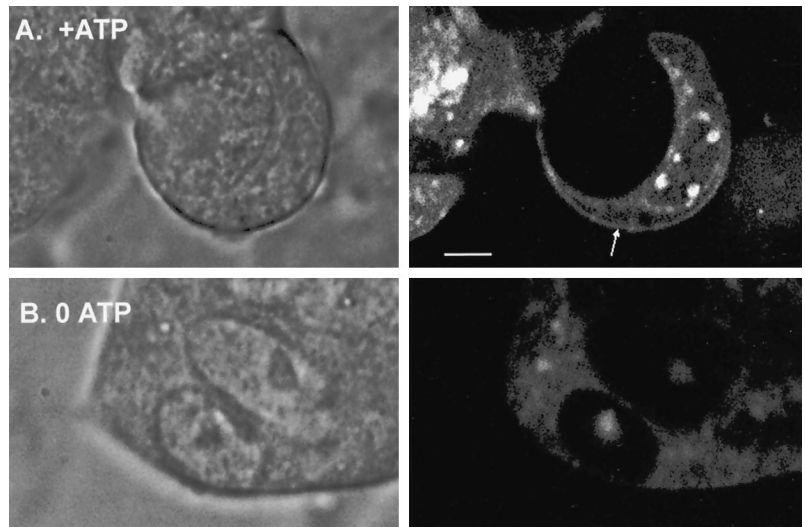
FIG. 4. **Effects of angiotensin II and DMPP on the localization of PH-GFP.** Cells in Ca^{2+}-containing PSS were stimulated for the indicated times with either angiotensin II (0.1 μM, *panel A*) or DMPP (20 μM, *panel B*). There was a transient increase in cytosolic PH-GFP at 10 s with both angiotensin II and DMPP. DMPP also induced regions of PH-GFP accumulation on the plasma membrane (*arrows*). The calibration *bar* corresponds to 5.7 μm in *A* and 5 μm in *B*.

stimulated with Ba^{2+}. Millimolar Ba^{2+} is a strong secretagogue that stimulates exocytosis without activating phospholipase C (51, 52). Ba^{2+} caused only small increases in cytosolic PH-GFP. In four of five cells, the increase in cytosolic PH-GFP was 20% or less. In only one cell was the increase 50%. The average maximal increase caused by Ba^{2+} was $19 \pm 7\%$ ($n = 5$) compared with $93 \pm 30\%$ ($n = 5$, $p < 0.05$) by DMPP and $270 \pm 95\%$ ($n = 3$, $p < 0.02$) by angiotensin II. The smaller increases in cytosolic PH-GFP induced by Ba^{2+} suggest that the larger shifts caused by angiotensin II and DMPP reflect competition for PH-GFP between cytosolic inositol polyphosphates and plasma membrane PtdIns-4,5-P$_2$. The small shifts caused by Ba^{2+} may reflect dynamic changes in the plasma membrane caused by plasma membrane addition by exocytosis.

Expression of PH-GFP Inhibits Exocytosis—If PtdIns-4,5-P$_2$ in the plasma membrane is required for exocytosis, then PH-GFP by binding plasma membrane PtdIns-4,5-P$_2$ may inhibit secretion. The following experiments address this issue using a variety of different methods of stimulating and measuring exocytosis in PH-GFP-expressing chromaffin cells.

Chromaffin cells were co-transfected with plasmids encoding hGH and PH-GFP or with a plasmid encoding hGH and a control plasmid (pCMVneo). The transiently expressed hGH is a soluble constituent of chromaffin granules in the transfected cells, and its release is a quantitative measure of chromaffin granule exocytosis (44). The effects of PH-GFP on secretion stimulated by DMPP or by Ba^{2+} were investigated (Fig. 7, *A* and *B*). The expression of PH-GFP inhibited secretion induced by either DMPP or Ba^{2+} by 50% or 42%, respectively.

It is possible that the inhibition by PH-GFP of secretion induced by DMPP or Ba^{2+} was not a direct inhibition of the

secretory machinery, but rather an inhibition of Ca^{2+} (with DMPP stimulation) or Ba^{2+} influx. To more directly stimulate secretion, secretion was examined from digitonin-permeabilized cells (Fig. 7*C*). Because PH-GFP fluorescence decreased upon permeabilization (data not shown), probably because of efflux from the cells, Ca^{2+} was present together with digitonin to stimulate the cells immediately upon permeabilization. Secretion stimulated by 30 μM Ca^{2+} was inhibited by 50%. PH(S34T,R40L)-GFP, which does not bind PtdIns-4,5-P$_2$, did not alter the secretory response. Expression of the PH-GFP also doubled hGH release in the absence of Ca^{2+}, an effect not observed with PH(S34T,R40L)-GFP.

The effects of PH-GFP on secretion were also investigated using patch-clamp techniques. Chromaffin cells were repetitively depolarized from -90 mV to $+20$ mV and membrane capacitance increases and Ca^{2+} current were measured (Fig. 8). Capacitance increases reflect the insertion of chromaffin granule membrane into the plasma membrane upon exocytosis. A relationship between the cumulative capacitance increase and *total* Ca^{2+} influx has been demonstrated by Nowycky and colleagues (53, 54). Virtually the identical quantitative relationship was observed in control transfected cells in our experiments (Fig. 8*A*, *open circles*) and in the previous work (Fig. 8*A*, *dashed line*). There were two effects of PH-GFP expression. First, the expression of PH-GFP inhibited the capacitance increase caused by a given Ca^{2+} influx by approximately 50%. This was observed over a wide range of Ca^{2+} influx (Fig. 8*A*) and was independent of pulse number (Fig. 8*B*). Second, the Ca^{2+} currents were reduced with the cumulative Ca^{2+} influx in the presence of PH-GFP approximately 70% that of control by the eighth depolarization. This change in Ca^{2+} influx did not
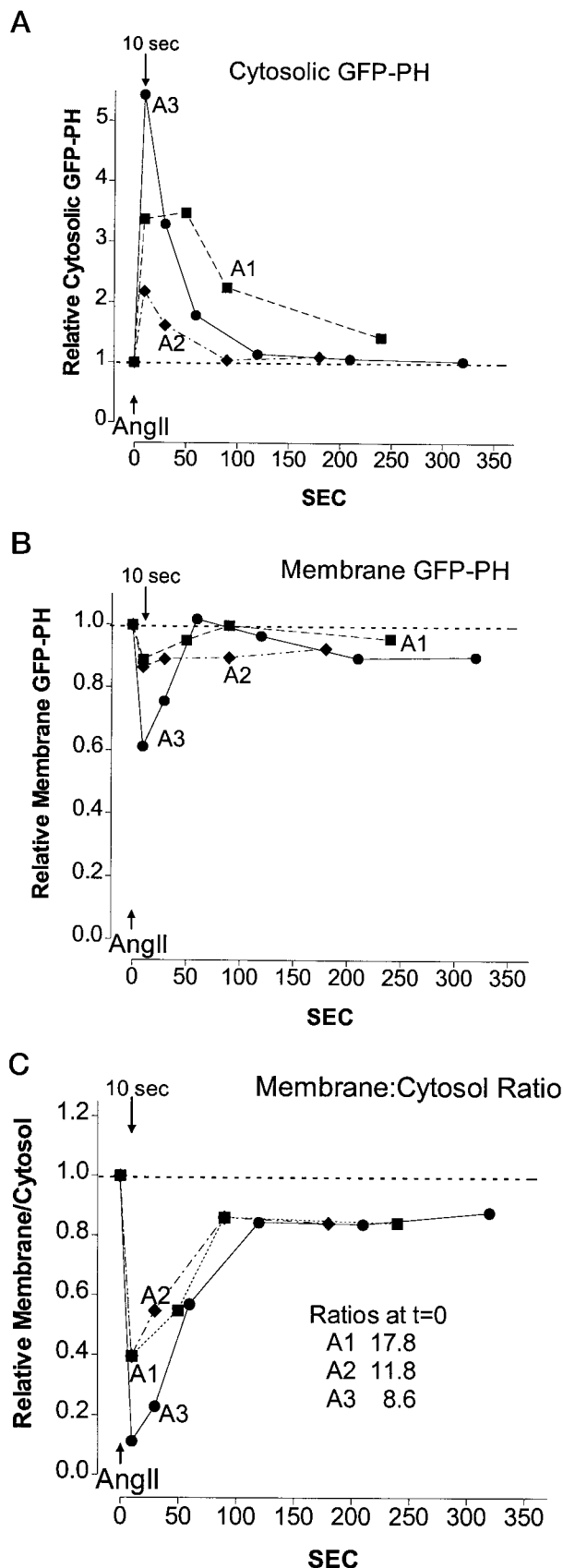
FIG. 5. **Changes in localization of PH-GFP upon stimulation with angiotensin II.** The fluorescence of PH-GFP was quantitated in the cytosol (*A*) and a segment of the plasma membrane (*B*) in three cells (A1, A2, and A3) stimulated with angiotensin II (0.1 μM). The membrane:cytosol ratios were determined and normalized to 1 at time 0 for each cell. The time courses of changes in the relative ratios is presented in *C*. Cell A2 is shown in Fig. 4*A*.
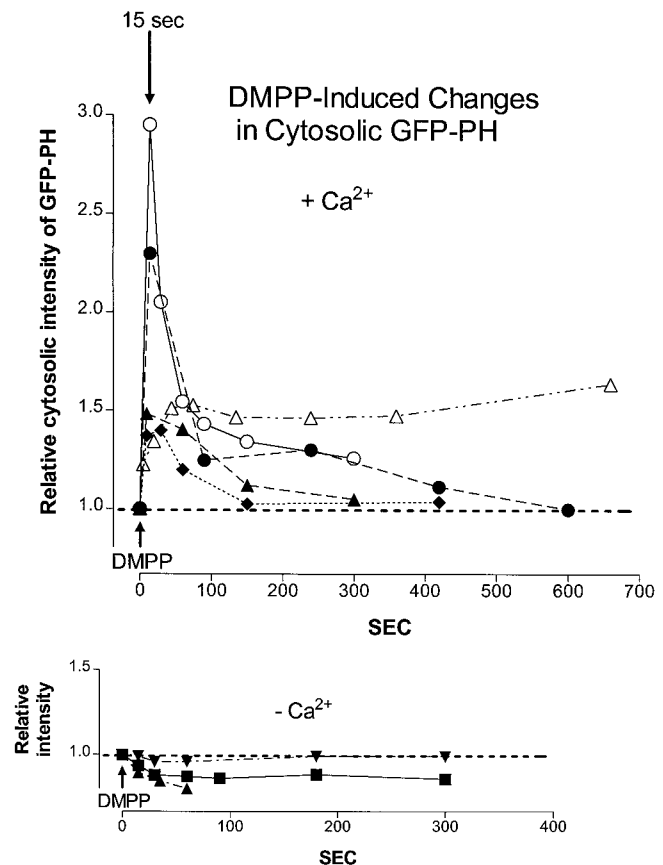

FIG. 6. **Changes in localization of PH-GFP upon stimulation with DMPP.** The time course of DMPP-induced changes in cytosolic PH-GFP is shown in cells incubated in the presence of Ca^{2+} (2.2 mM, 5 cells) or 0 Ca^{2+} (with 1 mM EGTA, 3 cells). The cell denoted by the *triangles* in *panel A* is shown in Fig. 4*B*.

TABLE I

Effects of angiotensin II and DMPP on the polyphosphoinositides

Chromaffin cells were incubated for 2 h with [^{32}P]phosphate, washed and either incubated with 100 nM angiotensin II or 20 μM DMPP for 15 s or incubated for an additional 5 min in physiological salt solution and then incubated with 100 nM angiotensin II or 20 μM DMPP for 2 min. [^{32}P]PtdIns-4,5-P$_2$ and [^{32}P]PtdIns-4-P are normalized to the amounts of [^{32}P]PtdIns.

	PtdIns-4,5-P$_2$/PtdIns	PtdIns-4-P/PtdIns
	%	*%*
Control (15 s)	21.3 ± 0.4	17.9 ± 0.6
Angiotensin II (15 s)	16.4 ± 0.4[a]	15.6 ± 0.33[b]
DMPP (15 s)	21.1 ± 0.7	18.6 ± 0.4
Control (2 min)	18.1 ± 0.5	15.4 ± 0.1
Angiotensin II (2 min)	18.1 ± 0.5	17.6 ± 0.3
DMPP (2 min)	23.2 ± 0.5[c]	23.2 ± 0.5[c]

[a] $p < 0.001$ *vs.* control at 15 s.
[b] $p < 0.02$ *vs.* control at 15 s.
[c] $p < 0.001$ *vs.* control at 2 min.

reflect a change in the kinetics of Ca^{2+} channel opening or closing. Each current trace of the first and eighth pulses (P1 and P8) was normalized to its maximal current and the traces averaged. The normalized traces from cells expressing PH-GFP superimposed on those from control-transfected cells at both P1 and P8 (Fig. 8*A*, *inset*).

DISCUSSION

Kinetically distinct steps can be distinguished in the secretory response from neuroendocrine cells, with slow ATP-dependent priming steps preceding the triggering of exocytosis by Ca^{2+} (12, 13, 55, 56). Two ATP-dependent steps have been
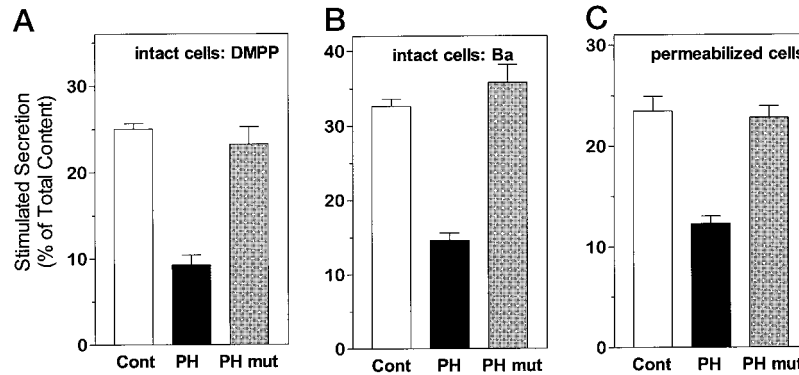
FIG. 7. **Expression of PH-GFP inhibits hGH secretion in transfected intact and permeabilized chromaffin cells.** Chromaffin cells were transfected with plasmids for hGH (*pXGH5*) and either the PH domain, the PH domain mutant PH(S34T, R40L)-GFP (*PH mut*), or pCMVneo (*Cont*) by calcium phosphate precipitates as described. Four days later intact cells were stimulated for 2 min with 20 μM DMPP in Ca^{2+}-containing PSS (*panel A*), or stimulated for 6 min by 2.2 mM extracellular barium (*panel B*). Cells in *panel C* were permeabilized for 3 min in KGEP containing 20 μM digitonin with or without 30 μM free calcium. The amount of hGH released into the medium and the amount remaining in the cells was determined. Release in the absence of stimulation was subtracted. Unstimulated release was 1.9% of total cellular content in *panel A*, 2.4% in *panel B*, and in 3.7% in control and PH mutant groups in *panel C*. Unstimulated release in permeabilized PH-GFP-transfected cells (*panel C*) was 7.6%. $n = 4$ wells/group.
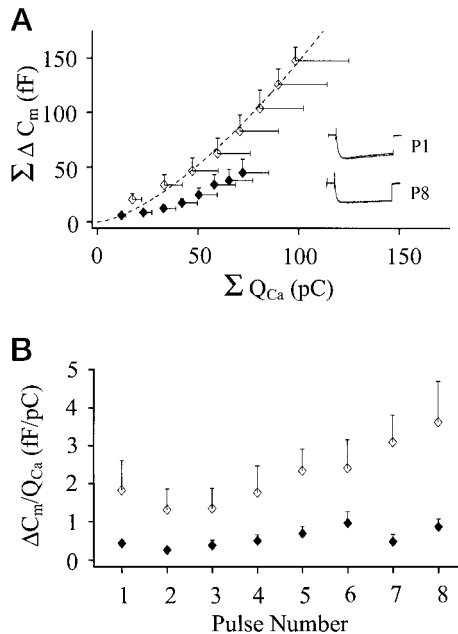
FIG. 8. **Expression of PH-GFP inhibits secretion measured electrophysiologically.** Chromaffin cells were co-transfected with plasmid encoding PH-GFP (8 cells, *filled symbols*) or control plasmid (pCMV.neo, 5 cells, *open symbols*) and a plasmid encoding ANP-GFP (to identify chromaffin cells). Experiments were performed the next day. The stimulus protocol consisted of a train of eight-step depolarizations (-90 mV to $+20$ mV, 50-ms duration) with an interpulse interval of 200 ms. *A*, cumulative capacitance change ($\Sigma\Delta C_m$) is plotted *versus* cumulative Ca^{2+} influx (ΣQ_{Ca}). *Dashed line* represents the standard relationship [$\Delta C_m = 0.147 \times (\Sigma Ca^{2+})^{1.5}$] described by Ref. 53. The *inset* compares the normalized Ca^{2+} currents for control and PH-GFP-expressing cells for the first and eighth pulse. The normalized traces overlap, indicating that PH-GFP did not affect the Ca^{2+} current kinetics. *B*, change in capacitance divided by Ca^{2+} influx ($\Delta C_m/Q_{Ca}$) for each pulse. Cells expressing the PH-GFP had significantly smaller $\Delta C_m/Q_{Ca}$ ($p < 0.0001$, unpaired Mann-Whitney test).

identified that occur seconds to minutes before the triggering of exocytosis by Ca^{2+}. One involves α-SNAP and N-ethylmaleimide-sensitive factor (NSF), which regulates the interaction and conformation of SNAREs (57–59). The other is the maintenance of PtdIns-4,5-P$_2$ through lipid kinases (11, 15, 16). This latter effect of ATP is responsible for at least 70% of the ATP-dependent secretion evident in digitonin-permeabilized chromaffin cells (11). In the present study, we visualized plasma

membrane PtdIns-4,5-P$_2$ in chromaffin cells and provide evidence that this pool of PtdIns-4,5-P$_2$ is involved in exocytosis.

PH δ_1 Identifies Plasma Membrane PtdIns-4,5-P$_2$ in Chromaffin Cells—The PH domain of phospholipase Cδ_1 binds to PtdIns-4,5-P$_2$ in membranes and is responsible for the tethering of the enzyme to its membrane site of action. It has previously been demonstrated that a fusion protein of this domain with GFP permits visualization of PtdIns-4,5-P$_2$ in a variety of cells (39, 40). When transiently expressed in chromaffin cells, PH-GFP almost exclusively labeled the plasma membrane without detectable labeling of chromaffin granules. A small amount was sometimes observed on the nuclear membrane, and low concentrations were in the cytosol in unstimulated cells. This labeling pattern is specific for active PH domain. A mutant, PH(S34T,R40L)-GFP, which is unable to bind to PtdIns-4,5-P$_2$, was well expressed but completely cytosolic.

Experiments with rhodamine-neomycin confirmed that PtdIns-4,5-P$_2$ is associated with the plasma membrane. Neomycin binds PtdIns-4,5-P$_2$ *in vitro* (41–43) and binds PtdIns-4,5-P$_2$ in chromaffin cells (11). Rhodamine-neomycin, although less specific than PH-GFP in its associations in the cell, labeled the plasma membrane in permeabilized cells in the presence of ATP. It is likely that rhodamine-neomycin labeled plasma membrane PtdIns-4,5-P$_2$ because labeling was much less evident when PtdIns-4,5-P$_2$ was permitted to decline by permeabilizing cells in the absence of ATP (11). Rhodamine-neomycin also labeled nuclear structures, perhaps reflecting the presence of nuclear PtdIns-4,5-P$_2$ (60). PH-GFP only infrequently labeled intracellular, nonnuclear, punctate structures.

Chromaffin granules were labeled with neither PH-GFP nor rhodamine-neomycin. It is possible that an unknown plasma membrane protein ligand for PH δ_1 together with PtdIns-4,5-P$_2$ caused preferential binding of PH-GFP to the plasma membrane. Alternatively, PH-GFP, because of its large size, may not be able to bind to chromaffin granule membrane PtdIns-4,5-P$_2$ because of limited accessibility. However, rhodamine-neomycin binding to PtdIns-4,5-P$_2$ is unlikely to be limited in the same manner. Although the data are not conclusive, they suggest that the chromaffin granule membrane may contain significantly less PtdIns-4,5-P$_2$ than the plasma membrane. The findings are consistent with other data. Isolated chromaffin granules can convert 40% of their PtdIns to PtdIns-4-P in the presence of ATP with little or no synthesis of PtdIns-4,5-P$_2$ (61). Chromaffin granules isolated from permeabilized chromaffin cells that had been incubated in the presence of

$[\gamma$-^{32}P]ATP after sequential pharmacological inhibition and reversal of inhibition of PtdIns 4-kinase contained significant $[\gamma$-^{32}P]PtdIns-4-P but undetectable $[\gamma$-^{32}P]PtdIns-4,5-P$_2$ (62). Synaptic vesicles may also contain little PtdIns-4,5-P$_2$. A recent report indicates that, although PtdIns-4,5-P$_2$ is essential for budding from the plasma membrane and endocytosis in the exocytosis/endocytosis cycle of synaptic vesicles, the lipid must be dephosphorylated in order for the clathrin-coated endocytic vesicle to transform into a functional synaptic vesicle (63).

Plasma Membrane PtdIns-4,5-P₂ Is Important in Exocytosis—Although the data do not rule out the presence of PtdIns-4,5-P$_2$ on the chromaffin granule membrane, the specific labeling of plasma membrane PtdIns-4,5-P$_2$ by PH-GFP provided the opportunity to determine whether this pool of PtdIns-4,5-P$_2$ is the pool required for exocytosis. We used four different ways of stimulating exocytosis and in every case, the expression of PH-GFP significantly inhibited secretion. DMPP- or Ba^{2+}-induced secretion from intact cells expressing PH-GFP was inhibited by greater than 50%. Because the inhibition could have been caused by a PH-GFP-induced change in either Ca^{2+} or Ba^{2+} influx in the transfected cells rather than by a downstream effect on the secretory response, secretion from permeabilized cells in which the Ca^{2+} concentration is directly controlled was investigated. The expression of PH-GFP inhibited by 50% secretion directly stimulated by 30 μM Ca^{2+}. Finally, patch clamp measurements of capacitance increases (reflecting insertion of chromaffin granule membrane into the plasma membrane upon exocytosis), indicate that secretion was similarly inhibited by the expression of PH-GFP. Capacitance increases were reduced by 50% or greater for a given amount of Ca^{2+} influx. Although it is possible that the reduced increase in capacitance reflects more rapid endocytosis rather than reduced exocytosis, the data are consistent with the inhibition of secretion measured by biochemical means over a longer time. Taken together, these experiments strongly implicate an important role for plasma membrane PtdIns-4,5-P$_2$ in maintaining secretion in response to a Ca^{2+} signal.

The inhibition of secretion by transiently expressed PH-GFP is similar to previous results with other agents that bind PtdIns-4–5-P$_2$. Neomycin inhibits secretion from digitonin-permeabilized cells at concentrations that bind PtdIns-4,5-P$_2$ in cells (11, 64). A basic peptide derived from the C2b region of Rabphilin3 that binds PtdIns-4–5-P$_2$-containing lipid vesicles, but not a scrambled amino acid derivative, inhibits secretion from permeabilized chromaffin cells (31). These experiments with acute exposure to PtdIns-4,5-P$_2$-binding agents and the present experiments with long term exposure to a PtdIns-4,5-P$_2$-binding protein demonstrate a consistent inhibition of secretion.

The polyphosphoinositides including PtdIns-4,5-P$_2$ and 3-phosphorylated forms interact with and regulate numerous cytoskeletal proteins and proteins involved in vesicular trafficking (reviewed in Refs. 10 and 65). The requirement of plasma membrane in secretion may reflect a role for the lipid in regulating cytoskeletal dynamics immediately adjacent to the plasma membrane during exocytosis (66) or the function of proteins specifically involved in the exocytotic pathway. It is intriguing that there are now three proteins associated with the chromaffin granule membrane that bind PtdIns-4,5-P$_2$ in a specific manner: synaptotagmin (29), Rabphilin3 (31), and calcium-dependent activator protein for secretion (30). The Ca^{2+}-regulated interaction of one or more of the proteins with PtdIns-4,5-P$_2$ in the plasma membrane may modulate protein function and could possibly be directly involved in the fusion reaction.

The electrophysiological experiments revealed a second effect of expression of PH-GFP to reduce the magnitude of the Ca^{2+} currents. Ca^{2+} entry during each pulse was significantly reduced so that, by the end of the eighth pulse, the cumulative Ca^{2+} influx was approximately 30% lower (Fig. 8*A*). The smaller Ca^{2+} currents were not caused by PH-GFP-induced alteration in the kinetics of the Ca^{2+} current. Instead, they may reflect fewer Ca^{2+} channels opening upon depolarization. This phenomenon remains to be investigated.

Dynamics of PH-GFP Localization during Stimulation of Chromaffin Cells Probably Reflects the Activation of Phospholipase C—The characterization of PH-GFP in chromaffin cells revealed movement of PH-GFP from plasma membrane to cytosol upon stimulation with angiotensin II or DMPP. As described in other cell types (39), this movement probably reflects the activation of phospholipase C. The movement of PH-GFP was observed despite the possible reduction of phospholipase C activity in transfected cells due to the binding of PtdIns-4,5-P$_2$ by the PH domain.

Angiotensin II activates phospholipase C through a G protein-linked receptor. The nicotinic agonist DMPP activates phospholipase C through a rise in cytosolic Ca^{2+} caused by Ca^{2+} influx across the plasma membrane (51). Because highly effective mechanisms for maintaining the levels of the polyphosphoinositides in chromaffin cells prevented either stimulus from causing large decreases in PtdIns-4,5-P$_2$ (DMPP actually caused an increase in PtdIns-4,5-P$_2$; see Table I and Ref. 48), the translocation of PH-GFP to the cytosol probably did not result from decreased levels of PtdIns-4,5-P$_2$. Instead, translocation of PH-GFP may have occurred because of increases in cytosolic Ins(1,4,5)P$_3$. PH δ_1 has as least as high an affinity for Ins(1,4,5)P$_3$ as for membrane PtdIns-4,5-P$_2$ (35, 49, 50). The increased amounts of cytosolic Ins(1,4,5)P$_3$ would be expected to compete with membrane PtdIns-4,5-P$_2$ for PH-GFP.

Only a small part of the translocation is likely to be caused by membrane changes associated with exocytosis. The influx of Ca^{2+} induced by DMPP stimulates secretion of 20–30% of the total cellular catecholamine in contrast to the rise in cytosolic Ca^{2+} induced by angiotensin II which stimulates no more than 2% release (67).[2] DMPP was no more effective than angiotensin II in causing a redistribution of PH-GFP. Furthermore, Ba^{2+} influx, which is a strong stimulus for exocytosis, caused a much smaller increase in cytosolic PH-GFP than either angiotensin II or DMPP. Ba^{2+} is distinguished from angiotensin II and DMPP by *not* stimulating phospholipase C activity and an increase in Ins(1,4,5)P$_3$ in chromaffin cells (51, 52)

In summary, the characteristics of the rapid movement of PH-GFP to and from the plasma membrane upon stimulation of the cells supports the conclusion that the probe binds to the plasma membrane pool of PtdIns-4,5-P$_2$.

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